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Lignin-first biomass fractionation using a hybrid organosolv – Steam explosion pretreatment technology improves the saccharification and fermentability of spruce biomass

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ABSTRACT

For a transition to a sustainable society, fuels, chemicals, and materials should be produced from renewable resources. Lignocellulosic biomass constitutes an abundant and renewable feedstock; however, its successful application in a biorefinery requires efficient fractionation into its components; cellulose, hemicellulose and lignin. Here, we demonstrate that a newly established hybrid organosolv – steam explosion pretreatment can effectively fractionate spruce biomass to yield pretreated solids with high cellulose (72% w/w) and low lignin (delignification up to 79.4% w/w) content. The cellulose-rich pretreated solids present high saccharification yields (up to 61% w/w) making them ideal for subsequent bioconversion processes. Moreover, under high-gravity conditions (22% w/w) we obtained an ethanol titer of 61.7 g/L, the highest so far reported for spruce biomass. Finally, the obtained high-purity lignin is suitable for various advanced applications. In conclusion, hybrid organosolv pretreatment could offer a closed-loop biorefinery while simultaneously adding value to all biomass components.

1. Introduction

Increasing environmental concerns over the use of fossil resources, have accelerated the need for a switch from fossil to renewable resources for the production of fuels, chemicals, and materials (Matsakas et al., 2017). Owing to its abundance, utilization of lignocellulosic biomass for this purpose has attracted substantial attention during the last decades. Various sources of lignocellulose have been evaluated, including agricultural residues, forest residues, and energy crops (Katsimpouras et al., 2017). Lignocellulosic biomass derived from forest residues is an important renewable source of raw materials for countries such as Sweden, where forests cover 57% of the land and contribute significantly to the national economy (<https://www.sveaskog.se/en/forestry-the-swedish-way/short-facts/brief-facts-1/>).

The complex nature of lignocellulosic biomass, which consists of cellulose, hemicellulose, and lignin, requires a pretreatment process to remove the natural barriers of lignocellulose against microbial and

enzymatic attacks and make the cellulose easily hydrolysable. At the same time, the pretreatment conditions should minimize sugar degradation to inhibitory compounds (Kumar et al., 2009). Researchers have been actively focusing on the development of pretreatment methods including steam explosion, hydrothermal, and dilute acid treatment (Kataria et al., 2017; Nitsos et al., 2017). These methods effectively improve saccharification yields prior to microbial conversion and constitute the core of the so-called ‘gluco-centric’ approach, whose focus is the transformation of lignocellulosic carbohydrate fractions into fuels and chemicals. For bioethanol production, the emphasis is often on cellulose conversion and efforts to engineer *Saccharomyces cerevisiae* (the most commonly used fermenting microorganism) to consume pentose sugars, which it naturally cannot ferment (Becker and Boles, 2003). Lignin is normally collected at the end of pretreatment as a low-purity by-product and is often used as a combustion material for heat and power production owing to its high heating value (Parsell et al., 2013; Xu et al., 2014). Lignin is also a

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renewable and abundant source of aromatic compounds (Ragauskas et al., 2014), hence its valorization for fuel and chemical production is vital for the success of future biofuel industries (Beckham et al., 2016; Vardon et al., 2015).

A resource-efficient way to convert lignocellulosic biomass to fuel and chemicals is through biorefining. Lignocellulosic biomass is separated into its components (cellulose, hemicellulose, and lignin), which in turn are converted to fuels, chemicals, and materials through various (thermo)-chemical and biochemical processes (Cherubini, 2010). Crucial in this respect, is a switch from lignin as a low-value material for combustion to a source for the production of high added-value chemicals and biomaterials. To this end, recovery of high-purity lignin is paramount. Moreover, the obtained lignin should be devoid of extensive modifications, which often hinder its utilization (Parsek et al., 2013). Current pretreatment technologies fail to yield high-purity lignin and, given the harsh operating conditions, they lead to modifications of the lignin molecule (Parsek et al., 2013). An alternative approach is to recover lignin early in the process, prior to further processing of the carbohydrate fractions (Matsakas et al., 2018a). This strategy emphasizes the need for fractionation technologies capable of producing individual streams of cellulose, hemicellulose, and lignin enabling a wide portfolio of fuels, chemicals, and biomaterials (Bozell, 2010). Organosolv pretreatment/fractionation is considered one of the most promising methods for biomass delignification and fractionation, resulting in the production of relatively clean streams of cellulose, hemicellulose, and lignin (Matsakas et al., 2018b). This can be achieved by separating the cellulose-rich pretreated solids from the pretreated liquor by filtration, followed by recovery of the solvent through distillation leading to the precipitation of lignin, leaving behind an aqueous solution containing mainly the solubilized hemicellulose. Organosolv treatment employs a mixture of organic solvents (such as ethanol, methanol, or acetic acid), water, the presence or absence of a catalyst, and between 100 and 250 °C (Matsakas et al., 2018b; Sun et al., 2018). It generates three distinct streams, namely a cellulose-rich solid stream, a liquid stream containing solubilized hemicellulose, and a solid stream of high-purity lignin (Raghavendran et al., 2018). Additionally, the lignin produced in this way has low ash content, is sulfur-free, and retains the majority of β -ether bonds, maintaining a structure close to the natural one (Azadi et al., 2013; Matsakas et al., 2018b; Sun et al., 2018).

Owing to the high fractionation efficiency of organosolv pretreatment, we previously investigated its use on forest biomass (spruce and birch) (Nitsos et al., 2016). To further improve its fractionation efficiency, we established a hybrid pretreatment method, whereby the fractionation efficiency of conventional organosolv pretreatment was combined with the physical biomass size reduction caused by steam explosion (Matsakas et al., 2018b). This was achieved by including an explosive discharge step at the end of traditional organosolv cooking, thus merging the two methods into a single-step process. This novel hybrid method has already been proven for the pretreatment and fractionation of birch woodchips (Matsakas et al., 2018b). The main aim of the current work was to investigate the potential of applying the hybrid method to the pretreatment and fractionation of spruce (softwood) and study the effect of various parameters (duration, ethanol content, use of an acid catalyst) on the fractionation efficiency of the process. Furthermore, aiming at high yields of ethanol fermentation at low- and high-gravity, we determined the effect of the pretreatment conditions on the enzymatic saccharification of cellulose-rich pretreated solids and the fermentation performance.

2. Materials and methods

2.1. Feedstock

Bark-free wood chips of Norway spruce (*Picea abies* L.) were obtained from mills located in Northern Sweden. The chips were air-dried and milled through a 1-mm screen with a knife mill (Retsch SM 300,

Retsch GmbH, Haan, Germany) and stored at room temperature. The composition of untreated milled spruce (expressed per dry basis) was as follows: 37.6% w/w cellulose, 17.6% w/w hemicellulose, and 32.6% w/w lignin. Milled chips used during pretreatment trials had a moisture content of 6.2% w/w.

2.2. Pretreatment and lignin recovery

Pretreatment trials were performed in a steam explosion reactor modified to operate in an organosolv mode as described previously (Matsakas et al., 2018b). In each pretreatment trial, 200 g of milled chips were used. The chips were wetted with 400 g of ethanol (absolute ethanol, $\geq 99.8\%$), which also contained the acid catalyst (if applicable), prior to loading in the reactor. After closing the reactor door, the additional ethanol required to attain the desired content during pretreatment was loaded with an external pump. During all trials, temperature was maintained at 200 °C using a combination of electrical heating elements around the reactor and steam inside the reactor. When pretreatment was completed, the discharge valve was opened causing a rapid decompression of the reactor and the exploded slurry was collected through the cyclone (Matsakas et al., 2018b). The following process parameters were tested: treatment duration (15–60 min), ethanol content in the pretreated liquor (52–65% v/v), and addition of sulfuric acid as an acid catalyst (0–1% w/w_{biomass}).

After pretreatment, the slurry was collected and vacuum-filtered to separate the solids from the liquid. The liquor was collected, and the solids were washed with ethanol, air-dried, and stored at room temperature until further use. To reduce the solubility of lignin, ethanol was removed from the pretreatment liquor in a rotary evaporator and lignin was separated by centrifugation at 14,000 rpm (29,416 \times g) at 4 °C for 15 min. The clear liquor (containing the solubilized sugars) was collected for sugar determination, whereas the precipitated lignin was air-dried and stored at room temperature until further use.

To study the effect of the explosive discharge step on pretreatment, a control experiment without the inclusion of the explosive discharge step was also performed. Accordingly, instead of opening the discharge valve at the end of the pretreatment, the reactor was gradually depressurized by opening the valve to the blowout tank. The pretreatment liquid was removed through this valve, whereas the pretreated solids remained inside the reactor. The operational conditions employed in this control experiment were akin to the optimal conditions (52% v/v ethanol content for 30 min) without the use of the acidic catalyst. Finally, the hybrid organosolv – steam explosion pretreatment was compared to the traditional steam explosion pretreatment. Steam explosion (225 °C with 0.5% w/w H₂SO₄ for 5 min) of spruce wood chips was performed as described previously (Nitsos et al., 2017). Treatment without the explosive discharge step was performed under the same conditions as the steam explosion process and the reactor was depressurized analogously as during hybrid organosolv – steam explosion treatment.

2.3. Enzymatic saccharification trials

Pretreated solids were assessed for their hydrolysability using a commercial enzyme cocktail. Enzymatic saccharification was performed in 100 mL flasks (with cotton stoppers) containing 40 mL of 2% (w/v) dry solids in 50 mM citrate buffer (pH 4.8). The enzyme solution used in the current work was Cellic® CTec2 (Novozymes A/S, Bagsværd, Denmark) with an activity of 149 FPU/g (Wang et al., 2014). Based on our previous work on batch organosolv-pretreated spruce biomass (Raghavendran et al., 2018), the initial screening of pretreated solids obtained from the various pretreatment conditions was performed at an enzyme load of 22.5 FPU/g_{solids}. Subsequently, an enzyme dosage study was performed at loadings of 12, 22.5, 45, and 60 FPU/g_{solids} using the pretreated solids and the most promising pretreatment conditions. Enzymatic saccharification trials and sampling (at 0 and 48 h) were

performed as described previously (Matsakas et al., 2018b).

2.4. Ethanol fermentation at low- and high-solids concentration

Initially, low-gravity simultaneous saccharification and fermentation (SSF) was performed with the most promising material identified during the enzymatic saccharification trials, namely 52% ethanol, 30 min, and 0 or 1% w/w H₂SO₄ as described elsewhere (Matsakas et al., 2018b). Briefly, the pretreated solids were prehydrolyzed for 8 h at 6% w/w solid content by applying 18.5 FPU/g of enzyme load. Subsequently, the slurry was diluted to 5% w/w with the addition of *Saccharomyces cerevisiae* Ethanol Red® (20 mg dry cell mass/g_{solids}; Lesaffre Advanced Fermentations, Marcq-en-Baroeul, France) and nutrients (1 g/L yeast extract, 0.5 g/L (NH₄)₂HPO₄, and 0.025 g/L MgSO₄·7H₂O). The flasks were incubated at 35 °C and samples were withdrawn every day for 5 days. After centrifugation, the supernatant was collected for ethanol determination using high-performance liquid chromatography (HPLC). Prior to HPLC analysis, the supernatant was filtered through a 0.2 µm nylon filter.

Following low-gravity SSF trials, we evaluated the sample treated with acid catalyst under high-gravity SSF. Saccharification was performed at a solid content of 22% w/w in 50 mM citrate buffer (pH 4.8) for 8 h at 50 °C. Cellic® CTec2 was loaded at 18.5 FPU/g_{solids}. Due to the high-solids content, the slurry was very viscous with virtually no free water available, making proper mixing a challenge. To overcome this issue, saccharification was performed in a custom-made gravimetric saccharification chamber as described previously (Matsakas et al., 2014; Matsakas and Christakopoulos, 2013). Subsequently, when saccharification was completed, the slurry was collected and supplemented with nutrients (1 g/L yeast extract, 0.5 g/L (NH₄)₂HPO₄, and 0.025 g/L MgSO₄·7H₂O) from a concentrated stock solution, thus ensuring volume changes < 2% v/v. Finally, fermentation was initiated by inoculating 1 g/L (dry cell mass) of *S. cerevisiae* Ethanol Red® suspension, previously grown overnight in YPD medium at 35 °C and 180 rpm. Incubation was carried out at 35 °C and 120 rpm and samples were taken every day until no more ethanol was produced. The samples were diluted five times (mass basis), centrifuged to remove solids, and the supernatant was filtered through a 0.2 µm nylon filter prior to ethanol determination by HPLC (see Section 2.5).

2.5. Analysis

Chemical composition (cellulose, hemicellulose, and lignin) of untreated and pretreated solids was determined using the NREL protocol (Sluiter et al., 2012). Carbohydrates and acetyl groups were determined with an HPLC apparatus equipped with a refractive index detector. For carbohydrates, an Aminex HPX-87P column (BioRad, Hercules, CA, USA) was used and operated at 85 °C with ultrapure H₂O as the mobile phase at a flow of 0.6 mL/min. For acetyl groups (measured as acetic acid) determination was performed on an Aminex HPX-87H column (BioRad) operated at 65 °C with 5 mM H₂SO₄ as the mobile phase at a flow rate of 0.6 mL/min. Finally, inorganic ash was determined gravimetrically by treating the biomass at 550 °C for 3 h. Solubilization of cellulose, hemicellulose, and lignin in the pretreatment liquor following the pretreatment process was calculated by the following equation (Eq. (1)):

Component solubilization (%w/w)

$$= 100 \times \left(\frac{W_{untreat} \times x_{untreat} - W_{pretreat} \times x_{pretreat}}{W_{untreat} \times x_{untreat}} \right) \quad (1)$$

where $w_{untreat}$ and $w_{pretreat}$ are the dry masses of the untreated and recovered pretreated solids, respectively, and $x_{untreat}$ and $x_{pretreat}$ represent the component (cellulose, hemicellulose, or lignin) biomasses in % w/w. Isolated lignin fractions underwent the same procedure as untreated and pretreated solids to determine the carbohydrate and ash content.

Scanning electron microscopy (SEM) analysis was performed in a scanning electron microscope (7800-F Prime; JEOL, Tokyo, Japan) at high (10⁻⁴ Pa or lower) vacuum and an acceleration voltage of 3 kV. Prior to imaging, samples were mounted on conductive carbon tapes and coated with palladium.

Sugars present in the liquor (after ethanol removal and lignin precipitation) were determined by HPLC with the same method as described above. To enable the determination of oligosaccharides, these were hydrolyzed with the use of concentrated H₂SO₄ at a final concentration of 4%, followed by incubation at 121 °C for 1 h and neutralization with calcium carbonate. The sugars released during the enzymatic saccharification trials were determined as described above. The saccharification yield was calculated with the following equation (Eq. (2)):

$$\eta = 100 * \left(\frac{C_{glucose} * V_{liquid} * 0.90}{m_{solids} * x_{cellulose}} \right) \quad (2)$$

where $C_{glucose}$ represents the glucose concentration as determined by HPLC, V_{liquid} represents the volume of the liquid used in the saccharification, 0.90 is the correction factor for the conversion of cellulose to glucose, $x_{cellulose}$ is the mass fraction of cellulose (expressed in dry basis) and m_{solids} is the mass of dry solids. Ethanol production during low- and high-gravity SSF trials was determined as described for the quantification of acetic acid. Finally, the moisture content of the solid biomass was determined with a moisture analyzer (Sartorius MA 30, Sartorius AG, Goettingen, Germany).

3. Results and discussion

3.1. Evaluation of fractionation efficiency

3.1.1. Composition of pretreated solids

Based on our previous results on the pretreatment and fractionation of birch biomass using the newly developed hybrid reactor, we decided to apply the same temperature (200 °C) for the pretreatment of spruce biomass. Applying a fixed ethanol content, we first assessed the effect of treatment duration. Increasing the duration resulted in increased biomass solubilization from 35.5% (15 min treatment) to 47.2% (60 min treatment) due to extensive hydrolysis of biomass components (mainly hemicellulose and lignin) (Table 1). Extending the cooking time had a positive effect on cellulose content, as it led to an increase from 54.7% w/w (15 min) to 63.3% w/w (30 min) and 67.3% w/w (60 min) (Table 1). No significant solubilization of cellulose was observed with increasing cooking times. Instead, we observed a decrease in the hemicellulose content in pretreated solids, from 18.8% w/w at 15 min, to 15.4% w/w at 30 min and 9.8% w/w at 60 min, amounting to a hemicellulose solubilization of 70.8% for the 60 min pretreatment. Finally, lignin content first decreased from 24.9% w/w at 15 min to 16% at 30 min, but then increased back to 23.5% after 60 min, coinciding with reduced delignification from 30 to 60 min. This increase could be attributed to formation of pseudo-lignin (Bensah and Mensah, 2013; Ma et al., 2015), as observed also in our previous work on birch woodchip (Matsakas et al., 2018b). Pseudo-lignin are lignin-like insoluble compounds arising from the degradation of hemicellulose, and are detected as lignin during analysis (Kumar et al., 2013).

Based on the above observations, a duration of 30 min was used for further treatments, as it resulted in the lowest lignin content but a reasonably high cellulose content (63.3% w/w).

In the next stage, the effect of ethanol on biomass solubilization and composition was studied at high (65% v/v) and low ethanol (52% v/v) content. A decrease from 65 to 52% ethanol, increased biomass solubilization from 42 to 47.9% due to extended hemicellulose cleavage and increased delignification. This resulted from the acid-catalyzed cleavage of α - and β -ether bonds brought by higher water chemical activity (Pan et al., 2006), causing solubilization of hemicellulose to

Table 1
Recovery of pretreated solids obtained from different pretreatment conditions and their composition in cellulose, hemicellulose and lignin.

Pretreatment conditions			Biomass solubilization (% of initial biomass)	Cellulose (% w/w)	Hemicellulose (% w/w)	Lignin (% w/w)
Effect of time	65% v/v	15 min	35.5	54.7 (6.1)	18.8 (31.0)	24.9 (50.8)
		30 min	42.0	63.3 (2.3)	15.4 (49.3)	15.9 (71.7)
		60 min	47.2	67.3 (5.4)	9.7 (70.8)	23.5 (61.9)
Effect of ethanol	30 min	65% v/v	42.0	63.3 (2.3)	15.4 (49.3)	15.9 (71.7)
		52% v/v	47.9	66.0 (8.5)	6.0 (82.1)	14.9 (76.2)
Effect of catalyst	30 min – 52% v/v ethanol	0%	47.9	66.0 (8.5)	6.0 (82.1)	14.9 (76.2)
		0.2%	49.3	66.7 (10.0)	6.1 (82.3)	18.1 (71.8)
		1%	56.3	72.0 (16.3)	4.0 (90.2)	15.4 (79.4)

All results are expressed in dry basis. Numbers in parenthesis represent the mass fraction of each component (i.e., cellulose, hemicellulose, and lignin) that was solubilized at the end of pretreatment (calculated using Eq. (1)). Compositional analysis was performed in duplicates.

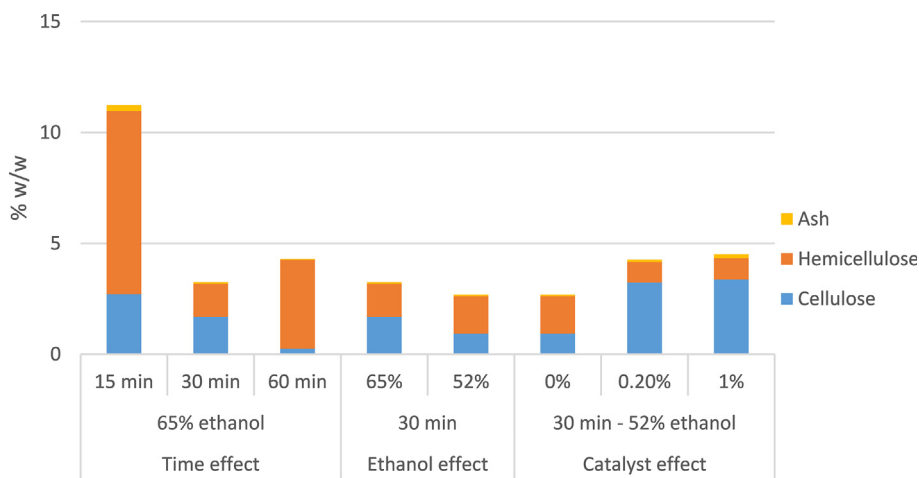


Fig. 1. Impurities content, both carbohydrates (cellulose and hemicellulose) and inorganic ashes of the lignin fractions obtained from various treatment conditions. The analysis was performed in duplicates, and the standard error was < 10% of the value.

increase from 49.3 to 82.1% and that of lignin from 71.7 to 76.2% (Table 1). The increased solubilization of hemicellulose and lignin coincided with a higher cellulose content (66% w/w), significantly reduced hemicellulose content (6% w/w), and essentially unchanged lignin content (14.9% w/w) (Table 1). Decreasing ethanol content had a more pronounced impact on hemicellulose solubilization, because it led to increased water content, which promotes the acid hydrolysis of hemicellulose. In contrast, delignification is a more complex process as it derives from the combined effect of lignin ether bond cleavage (promoted by higher water content) and lignin solubilization (promoted by higher ethanol content) (Pan et al., 2006). Therefore, reduced ethanol content led to increased aryl ether cleavages of lignin molecules; however, lignin removal was limited by lack of solvent for its dissolution (Ni and Hu, 1995). By reducing the ethanol content in the liquor, lignin cleavage is increased while lignin solubility is reduced. These two opposing effects counteract each other to the point whereby no significant decrease in lignin content is observed.

The introduction of sulfuric acid at 0.2 and 1% increased biomass solubilization to 49.3 and 56.3%, respectively. Addition of the acid increased the solubilization of hemicellulose to 90.2% and that of lignin to 79.4% (Table 1). It also increased the cellulose content of pretreated solids, which reached 72% w/w. The cellulose content achieved during this work was higher than the one obtained in our previous work (69.07% w/w), while at the same time, lignin content was considerably lower (15.4% w/w vs 25.03% w/w) (Nitsos et al., 2016). The present elevated delignification represents an improvement over other organosolv pretreatment methods, whereby maximum delignification (~65%) required substantially higher temperatures (~235 °C) (Agnihotri et al., 2015). Similarly, the high cellulose content in

pretreated solids obtained here surpasses other pretreatment methods, such as alkaline, ionic liquids, alkaline oxidation, steam explosion, dilute acid, and sulfur dioxide-catalyzed steam explosion (Kallioinen et al., 2013; Shafei et al., 2010; Shuai et al., 2010; Wang et al., 2018; Zhao et al., 2008). This demonstrates the efficiency of the proposed hybrid organosolv – steam explosion method for the pretreatment of spruce biomass.

3.1.2. Lignin purity

High delignification yields represent an important parameter for the selection of appropriate operational conditions of the organosolv fractionation. Another factor that determines the use of isolated lignins in advanced applications is their purity. Most technical lignins (e.g., kraft, lignosulfonates) contain various amounts of impurities, such as sugars, ash, and sulfur, which need to be removed prior to chemical or biochemical conversion processes (Vishtal and Kraslawski, 2011). Sulfur-free lignins are extremely versatile in many applications, such as thermosets and resins (Lora and Glasser, 2002). Organosolv-isolated lignins offer an excellent alternative to technical lignins, as they are both sulfur-free and of high purity (Zhao et al., 2009). Indeed, all lignin isolated in the present study contain very low or virtually no inorganic ash under all fractionation conditions (Fig. 1). The highest ash content (0.27% w/w) was recorded in lignin isolated with 65% v/v ethanol for 15 min, whereas the majority of lignins displayed an ash content < 0.1% w/w.

Impurities derived from sugars were also kept at low levels, not exceeding 4.3% w/w, except when lignin was isolated with 65% ethanol for 15 min, in which case they reached 11% w/w. The cellulose content was relatively low in all lignins, varying between 0.3 and 3.4%

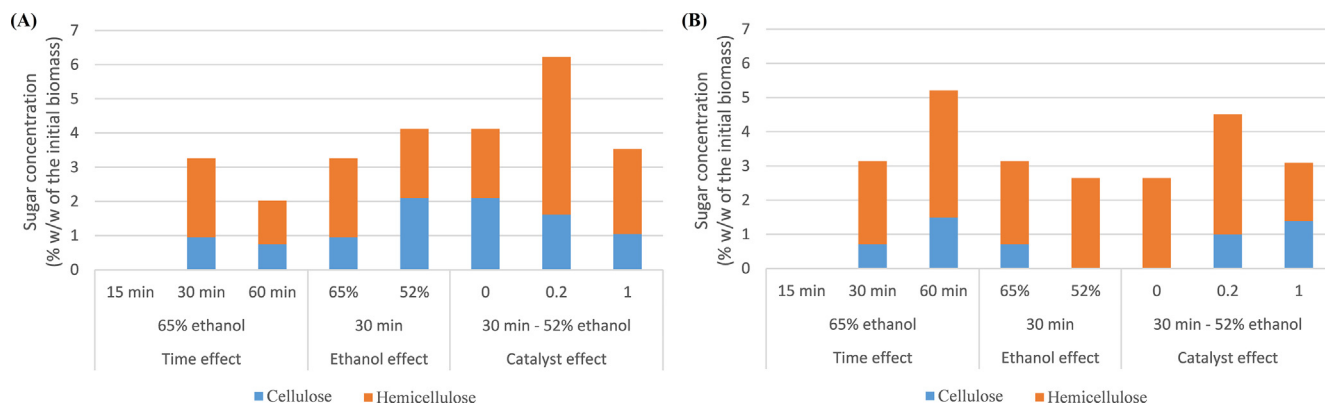


Fig. 2. Sugars of cellulose and hemicellulose origin found in the liquid fraction after lignin recovery, either as sugar monomers (A) or oligosaccharides (B). Results are expressed as grams of sugars (monomer or oligosaccharides) released from 100 g of biomass. The analysis was performed in duplicates.

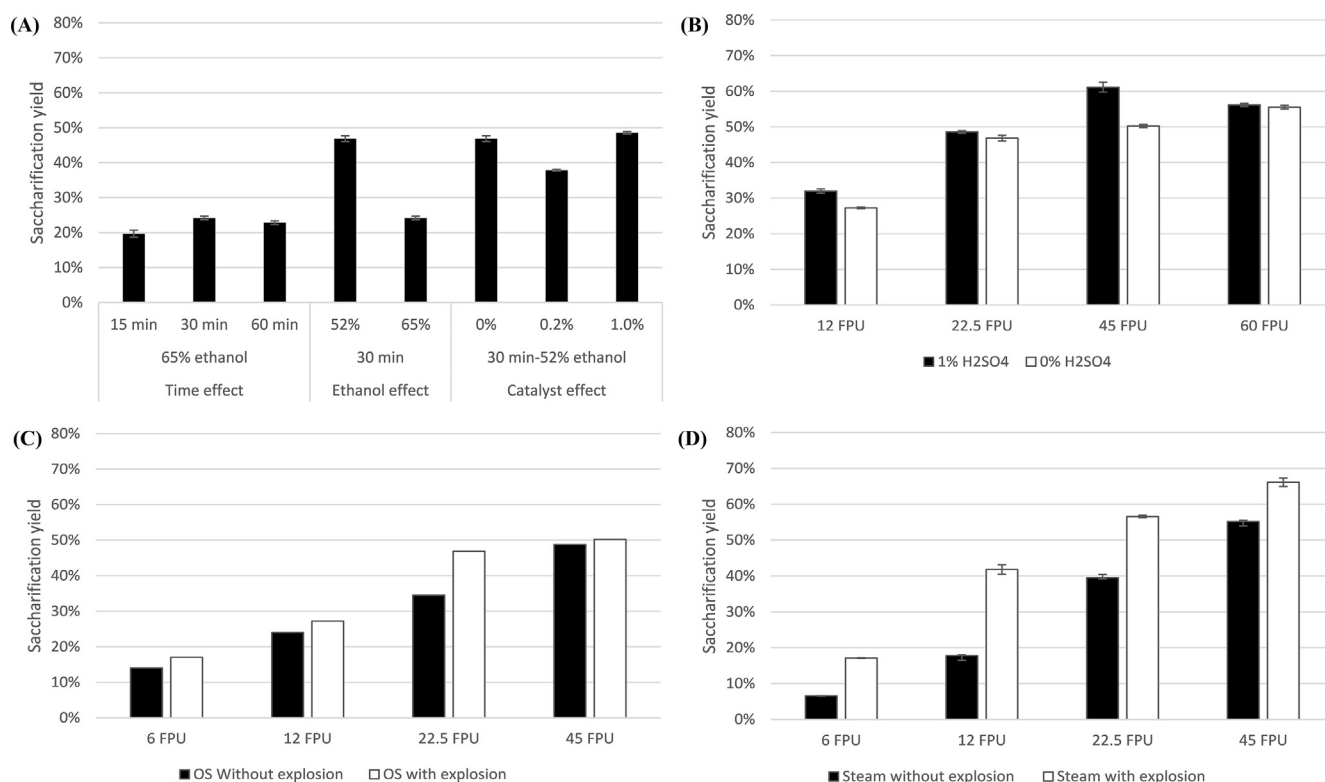


Fig. 3. (A) Enzymatic saccharification of hybrid organosolv-pretreated spruce biomass at 2% solids loadings using 22.5 FPU/g of enzyme preparation. (B) Enzyme dosage studies at 2% solids loadings with and without acid catalyst and with explosion. (C) Effect of enzyme dosage on organosolv-pretreated samples with and without explosive discharge. (D) Effect of enzyme dosage on steam explosion-treated samples with and without explosive discharge. Data points of spruce pretreated with steam explosion with the explosive discharge were taken from Raghavendran et al. (2018). All the enzymatic saccharification trials were performed in duplicates.

Table 2

Composition of pretreated solids in cellulose, hemicellulose and lignin after organosolv and steam explosion pretreatment with and without the explosive discharge.

Pretreatment conditions		Cellulose (% w/w)	Hemicellulose (% w/w)	Lignin (% w/w)
Organosolv (200 °C – 52% ethanol – 30 min)	With explosion	66.0	6.0	14.9
	Without explosion	69.9	4.0	18.8
Steam explosion (225 °C – 5 min – 0.5% w/w H ₂ SO ₄)	With explosion	38.2	0	53.1
	Without explosion	41.9	0.3	55.4

Compositional analysis was performed in duplication.

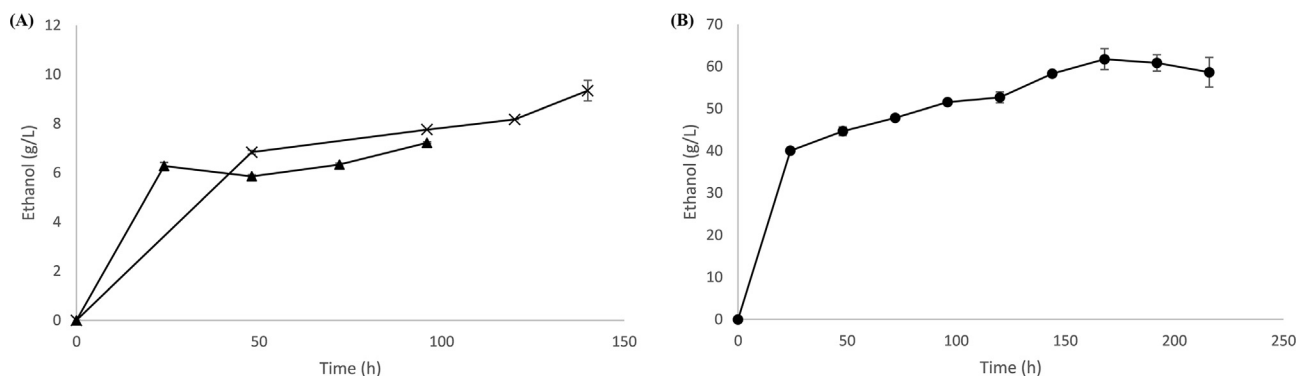


Fig. 4. Ethanol production profile during SSF of hybrid organosolv-steam explosion-pretreated spruce at (A) 5% w/w solids loading with (cross) and without (triangle) acid catalyst (1% w/w H_2SO_4) and at (B) 22% w/w solids loading with acid catalyst. The fermentation trials were performed in duplicates.

w/w across all cooking conditions (Fig. 1). Hemicellulose content, however, varied more between the tested conditions, reaching 8.3% w/w in the 15 min treatment, while remaining below 4% w/w in the other treatments. The 15 min peak could result from insufficient hydrolysis of hemicellulose-lignin bonds, causing parts of hemicellulose to remain attached to lignin and be isolated together. Alternatively, it could derive from the co-precipitation, during centrifugation, of lignin and high-molecular weight carbohydrates released by moderate hemicellulose hydrolysis at 15 min. Finally, introduction of the acid catalyst, even at the lower concentration, resulted in extensive hemicellulose cleavage and a hemicellulose content below 1% w/w.

3.1.3. Sugar recovery in the liquid fraction

Part of the sugars originating from cellulose and hemicellulose that are solubilized during pretreatment, can be recovered in the aqueous liquid fraction after ethanol removal and lignin precipitation. Notably, as shown in Fig. 2, no sugars were recovered in the liquid fraction after 15 min of treatment, in line with relatively low carbohydrate solubilization (Table 1) and their elevated presence in the lignin fraction (Fig. 1).

Sugars of cellulose origin were recovered mainly in the form of monomer (glucose) at a concentration between 0.7 and 2.1 g/100 g of initial solids (Fig. 2A). Glucose oligosaccharides (Fig. 2B) were below 1.5 g/100 g of initial solids, and no glucose oligosaccharides were detected following treatment with 52% v/v ethanol for 30 min. As expected, sugars of hemicellulosic origin, were generally more abundant owing to the more extensive hemicellulose solubilization (Table 1). Cooking time and ethanol content did not have any significant effect on total sugar concentration (sum of both monomeric sugars and oligosaccharides) recovered in the liquid fraction. Cooking time, however, influences the ratio between monomeric sugars and oligosaccharides, with increased cooking time favoring the latter. Finally, introduction of the acid catalyst at 0.2% w/ $w_{biomass}$ increased the overall recovery of hemicellulosic sugars in the liquid fraction (found mainly as sugar monomers). However, once the acid catalyst reached 1% w/ $w_{biomass}$, a significant drop in recovered hemicellulosic sugars was observed, possibly because of increased sugar degradation. A similar phenomenon was observed in our previous work using birch biomass (Matsakas et al., 2018b). This is important as hemicellulose-derived sugars can be further converted into fuels and chemicals through bioconversion and (thermo)chemical conversion routes (Delbecq et al., 2018; Saha, 2003).

3.2. Potential of pretreated solids for use in bioconversions

3.2.1. Evaluation of enzymatic saccharification efficiency

Efficient fractionation of lignocellulosic biomass is important as it allows the valorization of the various biomass fractions, with suitable and possibly different processes, in a biorefinery. Besides good fractionation yields, a key objective of any pretreatment method is to

generate solids that are readily converted to sugars during subsequent enzymatic saccharification. High sugar concentration is a prerequisite for increased ethanol titers during the bioconversion process. Fig. 3A shows the effect of cooking time, ethanol content, and catalyst addition on the saccharification yield. Cooking time did not significantly affect the yield, but a decrease in ethanol content from 65% to 52% favors higher yields. No difference in yield was observed with or without acid, however the yield decreased at 0.2% H_2SO_4 (w/w). Thus, a maximum yield of 49% was obtained with 52% ethanol for 30 min and 1% acid. To further assess the effect of acid catalyst, enzyme dosage studies were carried out. Presence of acid catalyst increased the yield at all enzyme dosages used (Fig. 3B), and particularly at 12 and 45 FPU/g, where it was 17 and 21% higher, respectively. In contrast, the traditional steam explosion process in the presence of acid catalyst (Raghavendran et al., 2018), exhibited a 24% and an 8% increase in the saccharification yields at the same enzyme dosages (12 and 45 FPU/g) studied. However, as the cellulose content in the hybrid treated spruce was considerably higher than the steam exploded (72% vs 38.2%); the saccharification yield per g of solids was 52% and 62% lower at those enzyme dosages for the steam exploded spruce, resulting in higher glucose production per gram of solids for the hybrid treated spruce.

3.2.2. Effect of explosive discharge on saccharification efficiency

The hybrid organosolv – steam explosion pretreatment method tested here on spruce biomass constitutes a unique combination of traditional organosolv and steam explosion methods. This combination was achieved by incorporating an explosive discharge step at the end of organosolv cooking. This allowed the fractionation efficiency of the organosolv to be complemented by the positive effect of the explosion on enzymatic saccharification yields (Matsakas et al., 2018b). Such positive effect has already been reported during steam explosion pretreatment of corn stover, beech wood, and spruce (Pielhop et al., 2016; Seidel et al., 2017). We were also the first to report that the explosive discharge step improved saccharification yields when included in the traditional organosolv fractionation of birch biomass (Matsakas et al., 2018b). Hence, here we examined whether the explosive discharge step had a positive impact on spruce biomass, as spruce and birch are characterized by significant structural differences and varying degrees of recalcitrance. Results were compared with the effect of the explosive discharge step during steam explosion pretreatment.

The chemical composition of pretreated solids derived from hybrid (with and without the explosive discharge step) and steam explosion (with and without the explosive discharge step) treatment is provided in Table 2. In all cases, the explosion step failed to have any major effect on the composition of pretreated solids. Cellulose, hemicellulose, and lignin content were comparable between the exploded and non-exploded samples, and the result was controlled by the chemical reactions, i.e., hemicellulose hydrolysis and lignin fragmentation, taking place before the explosive decompression of the mixture. Therefore, any

difference in the enzymatic hydrolysis of the solids was attributable to the explosion step. The explosive discharge had a clear positive impact on enzymatic digestibility of the solids, both in the case of traditional steam explosion treatment and in the hybrid process proposed in the current work (Fig. 3C and D). As for the latter, saccharification yield improved by between 3 and 21% (Fig. 3C), most notably at lower enzymatic loadings. Specifically, spruce treated with the explosive discharge achieved a saccharification yield of 47% at an enzyme load of 22.5 FPU/g, whereas 45 FPU/g was required to reach the same yield without explosion. The explosive discharge step had a profound positive impact on spruce treated with steam explosion (Fig. 3D). At a lower enzyme loading (< 12 FPU/g) the explosive discharge improved the saccharification yield by 2.6 times; however, as the enzyme load increased, the difference narrowed. In general, the solids treated with the explosive discharge had always superior enzymatic hydrolysis yields. Whereas these findings are in accordance with our previous results on birch biomass, it should be noted that the impact of the explosive discharge on saccharification yields is more significant for spruce biomass.

The samples were analyzed for their morphological characteristic using SEM and were imaged at both low and high magnification. SEM analysis revealed major differences between samples treated with the explosive discharge and without it. In the latter, distinct solid particles could be observed, their morphology seemingly intact and resembling that of untreated biomass (not shown). On the contrary, in samples subjected to explosive decompression, the particles appeared to have been destroyed, and consisted of a mass of smaller fragments with varying degrees of defibration.

3.3. Ethanol fermentation

Finally, we examined the potential of using the pretreated solids to produce ethanol. Initially, we tested the two most promising materials (namely treatment with 52% v/v ethanol for 30 min, with and without acid catalyst) identified by the enzymatic saccharification trials, under low-gravity conditions. The two pretreated solids resulted in similar saccharification yields, although the cellulose content was higher when the acid catalyst was included, and therefore appeared most suitable for ethanol fermentation. Ethanol production under low-gravity conditions is shown in Fig. 4A. The concentration of ethanol was higher when the acid catalyst was included, reaching 9.3 g/L, which equals 51% of the maximum theoretical yield.

Once the most promising material for ethanol fermentation was identified, we investigated its use under high-solids concentration. On the one hand, high-gravity fermentations are advantageous from an economic point of view, as they normally result in higher ethanol titers, better water economy, and more efficient processing (Koppram et al., 2014). On the other hand, under these conditions proper mixing of the slurry becomes a challenge resulting in poor contact of the enzymes with cellulose. To overcome this issue, we employed a freefall gravimetric mixing reactor as previously established (Matsakas et al., 2014; Matsakas and Christakopoulos, 2013). Spruce (pretreated with 52% v/v ethanol for 30 min and with 1% H₂SO₄ w/w_{biomass}) at a concentration of 22% w/w was prehydrolyzed for 8 h in this reactor. After this time, the concentration of glucose in the slurry reached 88 g/L, corresponding to a saccharification yield of 50%. The high amount of glucose at the onset of fermentation was very beneficial, with the ethanol concentration reaching 40 g/L within the first 24 h, which translated to a productivity of 1.67 g/L·h (Fig. 4B). Thereafter, fermentation continued for another 168 h, with ethanol reaching 61.7 g/L, or 68.6% of the theoretical maximum. While this ethanol concentration was lower compared to the ethanol content we previously obtained from birch (80 g/L) treated with the same method, it is, to the best of our knowledge, the highest reported in the literature for spruce. Indeed, an ethanol concentration of 40 g/L (20% solid content) and 45 g/L (10% solid content) were previously reported during high gravity saccharification and fermentation of steam pretreated spruce by using higher

enzyme load (22.5 FPU/g and 30 FPU/g glucan, respectively) (Bertilsson et al., 2009; Koppram and Olsson, 2014). The results obtained during this work were also higher in comparison to other woody materials, such as steam pretreated birch (14.4 g/L of ethanol, 20% solids content, 20 FPU/g), organosolv pretreated eucalyptus (42 g/L of ethanol, 15% solids content, 20 FPU/g) and acid bisulfite treated eastern redcedar (52 g/L of ethanol, 20% solids content, 46 FPU/g glucan) (Ramachandriya et al., 2013; Wang et al., 2014; Yáñez-S et al., 2013). It thus demonstrates the superiority of the recently established hybrid method for the treatment of spruce biomass. Despite the abundance of softwood, such as pine and spruce in the northern hemisphere, they are still recalcitrant to deconstruction compared to birch, which is a hardwood with very different lignin chemistry. Importantly, in the context of a biorefinery, addition of value to all streams and products that exit the refinery could compensate for the decreased productivity of some of the individual processes.

4. Conclusions

The newly established hybrid organosolv-steam explosion method efficiently fractionates spruce biomass and yields pretreated solids with high cellulose content (72% w/w), owing to efficient hemicellulose (90.2%) and lignin (79.4%) removal. Pretreated solids presented saccharification yields higher than those obtained from spruce treated with steam explosion. Low levels of inhibitors were generated, making the fermentation very efficient. Finally, a high ethanol concentration (61.7 g/L), corresponding to 68.6% of the theoretical maximum, was attained during high-gravity fermentation (22% w/w dry solids content).

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Appendix A. Supplementary data

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